Identification of novel antihypertensive peptides from wine lees hydrolysate

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23 **Abstract**

24 Enzymatic-assisted extraction using Flavourzyme has been demonstrated to be
25 a useful methodology to obtain wine lees (WL) enriched in phenolic compounds
26 and with enhanced antihypertensive activity. In addition to phenolic compounds,
27 hydrolysis of WL with Flavourzyme, which has hydrolase activity between others,
28 could release bioactive peptides. In this study, we investigate the presence of
29 antihypertensive peptides in the WL hydrolysate. Peptides were separated into
30 fractions by ultrafiltration and RP-HPLC. Next, peptide identification by nano-LC-
31 (Orbitrap)MS/MS was performed in the fractions with the highest angiotensin-
32 converting enzyme inhibitory (ACEi) activities. Six peptides were identified; three
33 of them showing ACEi (IC_{50}) values lower than 20 μM. The antihypertensive
34 effect of the peptides was evaluated in spontaneously hypertensive rats at an oral
35 dose of 10 mg/kg bw. Peptides FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA,
36 LDSPSEGRAPG and LDSPSEGRAPGAD exhibited antihypertensive activity,
37 confirming that they could contribute to the blood pressure-lowering effect of the
38 WL hydrolysate. These peptides have a great potential as functional ingredients
39 to manage hypertension.

40

41 ## 1. Introduction

42 Hypertension (HTN) is one of the main risk factor for cardiovascular diseases
43 (CVD), having a high prevalence in the global population (Mills et al., 2019). The
44 control of CVD risk factors and the reduction of the incidence of these diseases
45 have become a major global goal. In this regard, blood pressure (BP) reduction
46 is one of the strategies used to achieve these objectives (Unger et al., 2020). One
47 of the main mechanisms in regulating BP and vascular tone is the renin-
48 angiotensin-aldosterone system, in which the angiotensin-converting enzyme
49 (ACE) is a key player. ACE is considered an important target for the treatment of
50 HTN since the inhibition of its activity leads to BP reduction (Snauwaert et al.,
51 2017). Although synthetic ACE inhibitors are effective for HTN, it can exert
52 different adverse side effects in some patients (Alderman, 1996). Thus, natural
53 ACE inhibitors with antihypertensive properties are investigated as alternative to
54 synthetic drugs. Moreover, these compounds could be especially useful for pre-
55 hypertensive subjects, since this population is not usually clinically treated
56 (Margalef et al., 2017).

57 Peptides from dietary proteins exert a wide variety of functional activities such as
58 ACE inhibitory (ACEi), anti-inflammatory or antihypertensive activities
59 (Hernández-Ledesma, Del Mar Contreras, & Recio, 2011; Margalef et al., 2017;
60 Yoshikawa, 2015). Bioactive peptides can be released from the native protein
61 during food processing (fermentation or curing) or gastrointestinal (GI) digestion
62 (Toldrá et al., 2018). However, most of them are usually obtained by an
63 intentionally and controlled hydrolysis of food-derived proteins using specific
64 proteases or microorganisms (Toldrá et al., 2018). In the last decade, agri-food
65 industry by-products have emerged as an alternative protein source (Margalef et

66 al., 2017). In this regard, different by-products generated during the processing
67 of foods from vegetal and animal origin, have been successfully used to obtain
68 peptides with a wide range of bioactivities such as ACEi or antihypertensive
69 (Bravo et al., 2019; Margalef et al., 2017; Shobako et al., 2018; Toldrá et al.,
70 2020). This has led to the valorisation of these by-products allowing for their
71 recycling and making agri-food industries more environmentally friendly (Mora et
72 al., 2014).

73 Wine making is one of the most important agricultural activities in the world (258
74 mhL of wine estimated for 2020 harvest) (OIV, 2020). This activity produces tons
75 of by-products annually, including pomace (pulp, stems, seeds, skins), lees,
76 organic acids, CO_2 and water (Maicas & Mateo, 2020). Wine lees (WL) represent
77 between 2–6% of the produced wine volume and between 14-25% of all winery
78 by-products (Dimou et al., 2015, 2016). According to EEC regulation No. 337/79,
79 wine lees (WL) "is the residue formed at the bottom of recipients containing wine,
80 after fermentation, during storage or after authorized treatments, as well as the
81 residue obtained following the filtration or centrifugation of this product". WL
82 contain yeast cells, insoluble carbohydrates, organic acids, ethanol, phenolic
83 compounds, proteins, inorganic salts, lignin, pulp and other parts of the grape
84 (Jara-Palacios, 2019). In a recent study, our group reported that acute
85 administration of the soluble fraction of WL from grapes of Cabernet variety
86 exerted a potent antihypertensive effect in spontaneously hypertensive rats
87 (SHR) (López-Fernández-Sobrino, Soliz-Rueda, et al., 2021). Moreover, in other
88 study, enzymatic-assisted extraction was demonstrated to be a useful
89 methodology to maximize the release of phenolic compounds from WL and to
90 obtain extracts with enhanced functionalities. Specifically, WL hydrolysis was

91 performed with Flavourzyme®, which is a preparation widely used for protein
92 hydrolysis in industrial and research applications (Merz et al., 2015). After the
93 hydrolysis, in addition to phenolic compounds, a higher total amino acid residues
94 content was also observed in hydrolyzed WL compared to control WL, indicating
95 peptides or amino acid release. Therefore, it cannot be ruled out the involvement
96 of some of these peptides in the antihypertensive effect of this hydrolysate. In this
97 regard, different studies have reported generation of ACEi and antihypertensive
98 peptides from different protein sources after using Flavourzyme® (He et al., 2013;
99 Lee & Hur, 2017).

100 Therefore, considering all these evidence, the aim of this study was to investigate
101 the presence of antihypertensive peptides in the WL hydrolysate.

102 2. Materials and methods

103 2.1. Chemicals and reagents

104 Flavourzyme® 1000 L (EC 3.4.11.1, 500 LAPU/g from *Aspergillus oryzae*) was
105 kindly provided by Novozymes (Bagsværd, Denmark). Angiotensin converting
106 enzyme (ACE, EC 3.4.15.1) and the HPLC grade solvents acetonitrile and
107 trifluoroacetic acid were purchased from Sigma-Aldrich (Madrid, Spain). *O*-
108 aminobenzoylglicil-*p*-nitrofenilalanilprolina (o-Abz-Gly-p-Phe(NO_2)-Pro-OH) and
109 Captopril (PubChem CID: 44093) were provided by Bachem Feinchemikalien
110 (Bubendorf, Switzerland) and Santa Cruz Biotechnology (Dallas, TX, USA),
111 respectively. The peptides (FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA,
112 PAGELHP, LDSPSEGRAPG, LDSPSEGRAPGAD, purity grade ≥ 95 %) were
113 synthetized by Caslo Laboratory ApS (Caslo, Kongens Lyngby, Denmark). All
114 other chemical solvents used were of analytical grade.

115 ## 2.2. Wine lees hydrolysate

116 WL were kindly provided by the cellar Grandes Vinos y Viñedos S.A, located in
117 the Cariñena P.O.D area (Spain). They were collected after racking the red wine,
118 which was made from grapes of Cabernet variety. WL hydrolysate was carried
119 out following the method described by López-Fernández-Sobrino, Margalef, et
120 al., 2021. Briefly, enzymatic hydrolysis of WL was carried out with Flavourzyme®
121 (enzyme/substrate ratio, 80 LAPU/g protein) for 2 h at 25 ºC, pH 4.0 and 150 rpm
122 in a MaxQ Orbital Shaker (Thermo Fisher Scientific, Waltham, MA, USA).
123 Hydrolysis was stopped by lowering the pH of the solution to 3. Then, solution
124 was centrifuged at 3,000 × *g* for 15 min at 4 ºC and supernatant was collected
125 and kept at -20 ºC for further analysis. The soluble fraction of WL was also
126 obtained by centrifugation in the same conditions as a control (control WL).

127 Total protein content, measured by Kjeldahl method using 6.25 as factor,
128 hydrolysis degree, determined by TNBS method, and ACEi activity of hydrolyzed
129 WL were 0.69 ± 0.01 % (w/w), 7.61 ± 0.65 % and 0.63 ± 0.02 mg of dry weight/mL
130 (IC_{50} value), respectively (López-Fernández-Sobrino, Margalef, et al., 2021). All
131 analyses were done at least in triplicate.

132

133 ## 2.3. Isolation and identification of ACEi peptides from wine lees and the
134 hydrolysate

135 ### 2.3.1. Step I: Peptides separation based on their molecular weight and
136 hydrophobicity

137 First, the <3 kDa peptide fractions of control WL and hydrolysed WL were
138 obtained by ultrafiltration using a hydrophilic membrane with cut-off value of 3

kDa (Centripep, Amicon, Inc., Beverly, MA, USA). The obtained fractions were freeze-dried and kept at -20 ºC until use.

Regarding WL hydrolysate, peptides in the <3 kDa fraction (dissolved in Milli-Q water) were separated by a semipreparative reversed-phase liquid chromatography (RP-HPLC), using the same equipment and methodology described by Bravo et al., 2019 with some modifications. Specifically, in the current study, peptide separation was performed in gradient mode as follows: initial conditions 0 % B; 0-23.5 % B in 39.2 min; 23.5-90 % B in 9.8 min; 90-0 % B in 1 min. A 10 min post-run was required for column re-equilibration. Peptides were collected in different subfractions, which were freeze-dried and kept at -20 ºC until use. ACEi activity was determined in all subfractions (methodology described in 2.4 section) and the most active fractions were selected for peptide identification as it is described in section 2.3.2.

Non hydrolyzed WL was used as control and peptide identification was directly carried out using the <3KDa fraction as further peptide separation was not necessary.

2.3.2. Step II: Peptide identification by nano-LC-(Orbitap)MS/MS

Before mass spectrometry analysis, peptides in the <3kDa fraction of the control WL and in the RP-HPLC subfractions of the hydrolyzed WL (reconstituted in Milli-Q water) were purified by using an HLB SPE (Waters, USA) starting from 500 µL of sample, and eluted in 75:25 (v:v) acetonitrile: water with 0.1 % formic acid. The eluates were dried in a speedvac and resuspended in 50 µL 0.1% formic acid. Then, peptides were separated and analyzed by a nanoLC-(Orbitrap) MS/MS (LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, San José,

163 CA, USA). Peptides were separated by a 120 min acetonitrile gradient (A = water,
164 0.1% formic acid; B = acetonitrile, 0.1% formic acid) in a C18 reversed phase
165 (RP) nano-column (75 µm I.D.; 15 cm length; 3 µm particle diameter, Nikkyo
166 Technos Co. LTD, Japan) coupled to a trap nano-column (100 µm I.D.; 2 cm
167 length; 5 µm particle diameter, Thermo Fisher Scientific). The flow rate during
168 elution gradient was 300 nL/min.

169 For real time ionization and peptide fragmentation, an enhanced FT-resolution
170 spectrum (resolution = 30,000 FHMW) followed by a data dependent FT-MS/MS
171 scan from most intense ten parent ions with a charge state rejection of one using
172 a HCD fragmentation with a normalized collision energy of 35 % and dynamic
173 exclusion of 0.5 min.

174 For protein identification analysis, tandem mass spectra were extracted, and
175 charge state was deconvoluted by Proteome Discoverer version 1.4.0.288
176 (Thermo Fisher Scientific). All MS and MS/MS samples were analyzed using
177 Mascot (Version 2.5) search engine with three different nodes: 1) the proteome
178 of *Vitis vinifera* (29907 entries) from uniprot database, 2) the Swiss-Prot database
179 limited from fungi taxonomy (33712 entries) and 3) the common contaminants for
180 proteomic applications (247 entries). All these three searches assumed no
181 enzyme digestion and an error of 20 mmu for fragment ion mass and 10 ppm for
182 precursor ions. Oxidation of methionine and acetylation of N-termini were
183 specified as variable modifications, Visual verification of fragmentation spectra
184 was done for the identified peptides and only those found in both replicates were
185 considered. Identified peptides were chemical synthetized for further studies.

186 **2.4. Determination of ACEi activity**

187 ACEi activity was measured by a fluorescence technique according to López-
188 Fernández-Sobrino, Soliz-Rueda, et al., 2021. Specifically, λ_{ex} 360 nm, λ_{em} 400
189 nm and fluorescence measurements at 60 min (37ºC) were used to determine
190 ACEi activity, expressed initially as percentage of inhibition (%). Fractions or
191 peptides reconstituted in Milli-Q water, were tested at 0.11 mg of protein/mL and
192 0.83 mg of peptide/mL, respectively (concentration in the well). IC_{50} were also
193 calculated and expressed in µg/mL for RP-HPLC subfractions and in µM for the
194 synthetic peptides. Data are represented as a mean value of three determinations
195 ± SD.

196 For ACEi activity, protein content of HPLC subfractions were determined by the
197 bicinchoninic acid method (ThermoFisher Scientific) following the manufacturer's
198 instructions in a microplate format. A calibration standard curve was elaborated
199 with seroalbumin bovine. Determination of the protein content was performed at
200 least in duplicate. The results are expressed as the mean ± SD.

201 **2.5. Determination of antihypertensive activity**

202 Male SHR (17–20 weeks old, weighing 350–400 g) were obtained from Charles
203 River Laboratories España S.A. (Barcelona, Spain). Rats were singly housed in
204 a temperature-controlled animal quarter (22 ºC) with a 12 h light/dark period.
205 They were fed a standard diet based on chow Panlab A04 (Panlab, Barcelona,
206 Spain) and had free access to water.

207 After a 10-day adaptation period, animals were given a single dose of the
208 synthetic peptides dissolved in a volume of 1.5 mL in tap water by oral gavage
209 between 9 and 10 a.m. Synthesized peptides (FKTTDQQTRTTVA, NPKLVTIV,
210 TVTNPARIA, PAGELHP, LDSPSEGRAPG, LDSPSEGRAPGAD) were

211 administered to SHR at 10 mg/kg bw. Tap water and Captopril (50 mg/kg bw, a
212 known antihypertensive drug) were used as negative and positive controls,
213 respectively.

214 Systolic and diastolic blood pressure (SBP and DBP, respectively) were
215 measured using the tail-cuff method before and after 2, 4, 6, 8, 24, 48 and 72 h
216 of treatments administration (synthesized peptides, Captopril or water), according
217 to Quiñones et al., 2011. ΔSBP and ΔDBP were calculated as the difference
218 between the mean values of SBP or DBP after and before treatment
219 administration for each rat. Data were expressed as the mean values ± standard
220 error of the mean (SEM) for a minimum of six experiments.

221 The animal protocol followed in this study was conducted in accordance with the
222 European Communities Council Directive(86/609/EEC) and approved by the
223 Animal Ethics Review Committee for Animal Experimentation of the Universitat
224 Rovira i Virgili and by Generalitat de Catalunya (permission number 10522).

225 **2.6. *In silico* simulated peptides digestion**

226 *In silico* simulated peptide digestion was carried out using the program ExPASy
227 PeptideCutter, available at http://web.expasy.org/peptide_cutter. PeptideCutter
228 was used to predict the hydrolysis of peptide sequences identified in hydrolyzed
229 WL using the known enzymatic cleavage sites. Pepsin, chymotrypsin, trypsin,
230 lipase, and colipase were used to this purpose.

231 Moreover, BIOPEP data base was used to identify reported ACEi and
232 antihypertensive activities of the peptide-derived fragments obtained in the
233 simulated GI digestion of peptides (Minkiewicz et al., 2019).

234 **2.7. Statistical analysis**

235 Differences in the ACEi activity between peptides were analysed by one-way
236 analysis of variance (one-way ANOVA) and Tukey test as post hoc. Differences
237 between treatments in the *in vivo* studies were analysed by two-way ANOVA and
238 Tukey test as post hoc. All the analyses were performed using GraphPad Prism
239 7.04 for Windows (GraphPad Software, San Diego, California). Outliers were
240 determined by using Grubbs' test. Differences between groups were considered
241 significant when $p < 0.05$.

242 **3. Results**

243 **3.1. Identification of ACEi peptides**

244 WL hydrolysate was firstly subjected to ultrafiltration through a 3 kDa cut-off
245 membrane to isolate the low molecular weight peptides. Peptides in the <3 kDa
246 fraction were separated using a semipreparative RP-HPLC (conditions explained
247 in section 2.3.1). The obtained chromatogram showed a large number of peaks
248 (Figure 1A), indicating that the <3 kDa fraction is comprised of a complex mixture
249 of compounds. This fraction was divided into 9 RP-HPLC subfractions (named
250 from F.1 to F.9, Figure 1A), which were collected and lyophilized, and their ACEi
251 activities were measured (Figure 1B). Five subfractions showed an ACEi activity
252 higher than 50 % (F.4, F.6, F.7, F.8 and F.9) at 0.11 mg/mL. ACEi activity of these
253 selected fractions was also determined and expressed as IC_{50} values (µg of
254 protein/mL) (Figure 1C). The observed range of IC_{50} values were between 107
255 and 10 µg protein/mL, being the most active subfraction the F8 (12.3 µg of
256 protein/mL).

257 The five most active fractions were further analyzed by nanoLC-(Orbitap)MS/MS
258 to identify their peptides. Table 1 shows the six amino acid sequences identified
259 in those fractions, namely from P1 to P6. As an example, Figures S1A and S1B

260 show the MS/MS spectrum of the matched ions at m/z 543.267 and 636.298,
261 corresponding to the sequences LDSPSEGRAPG (P5) and LDSPSEGRAPGAD
262 (P6), respectively. These six peptides were chemical synthetized and their ACEi
263 activity was evaluated at a concentration of 0.83 mg/mL. ACEi activities ranged
264 from 2 to 95 % (Table 1). Peptides TVTNPARIA, PAGELHP and LDSPSEGRAPG
265 stood out for their high ACEi activity. Indeed, they showed an ACEi activity higher
266 than 70 % at the evaluated peptide concentration and with IC_{50} value lower than
267 20 μM. The amino acid sequence PAGELHP was the most active peptide with an
268 IC_{50} value of 0.5 μM.

269 In addition, peptides in the <3kDa fraction of the control WL was also analyzed
270 by nanoLC–OrbitapMS/MS. No peptides were identified.

271 **3.2. Antihypertensive activity of the synthetic peptides**

272 Antihypertensive effects of the six synthesized peptides were evaluated in SHR
273 (Figures 2 and 3). Before starting the study, SBP and DBP of animals were 198.9
274 ± 2.9 mmHg and 154.2 ± 3.8 mmHg, respectively, confirming hypertensive
275 conditions. As expected, BP of SHR receiving water did not significantly vary
276 throughout the study. Administration of Captopril (50 mg/kg bw) led to an
277 important decrease in SBP of SHR 2 h after its administration (Figure 2), reaching
278 the maximum decrease at 6-8 h post-administration (-44.0 ± 4.4 and 44.7 ± 2.9
279 mmHg, respectively). Its effect lasted until 24 h after its administration (-22.0 ±
280 5.7 mmHg of SBP).

281 Regarding peptides, treatment with peptides P1, P2, P3, P5 and P6 produced a
282 decrease in SBP of more than 10 mmHg (Figure 2). P1 and P6 were the most
283 effective peptides (Figures 2A and 2F). Specifically, their administration reduced

284 SBP from 2 h to 8 h post-administration, reaching the maximum values at 6 h
285 post-administration (-27.6 ± 2.2 and -24.3 ± 3.6 mmHg for P1 and P6,
286 respectively). P2, P3 and P5 also exerted an antihypertensive effect (Figures 2B,
287 2C, 2E). The maximum effect was also at 6 h post-administration, decreasing
288 SBP by -16.2 ± 2.0 to -19.0 ± 4.8 mmHg. In the case of the peptide P4, its
289 administration to the animals did not affect BP levels.
290 Regarding DBP, as expected, no differences were found in rats administered
291 water. Captopril produced the maxim BP drop by -47.2 ± 3.9 mmHg at 4h post-
292 administration (Figure 3). All peptides produced a decrease in DBP, except P4
293 that no presented differences with water-administered group. P1, P5 and P6 were
294 the peptides that showed the greatest antihypertensive effects, reducing DBP by
295 -32.0 ± 7.5, -27.0 ± 7.6 and -33.6 ± 3.6 mmHg at 6 h after peptide administration.

296 **3.4. *In silico* simulated gastrointestinal digestion of peptides**

297 The six peptides identified in hydrolyzed WL were subjected to an *in silico* study
298 to simulate their GI digestion. All the peptides were susceptible to be hydrolyzed
299 by gastric and/or intestinal proteases. Table 2 shows the prediction of amino acid
300 sequences released after FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA,
301 PAGELHP, LDSPSEGRAPG or LDSPSEGRAPGAD gastric and duodenal
302 digestion based on the *in silico* digestion approach. None of the obtained amino
303 acid sequences have been already reported in the data base BIOPEP to show
304 antihypertensive or ACEi activities. Only the amino acid sequences FK, IA and
305 HP, presenting in the extreme C-terminal of P1, P3, P4, respectively, were
306 previously reported to show an ACEi activity (Table 2).

307 **4. Discussion**

308 Bioactive peptides are small protein fragments encrypted in the protein, which
309 can exert a biological activity when they are released from the native protein
310 (Margalef et al., 2017). They can regulate important body functions through their
311 myriad activities, including antihypertensive, antimicrobial, antithrombotic,
312 immunomodulatory, opioid, antioxidant, and mineral binding functions
313 (Chakrabarti et al., 2018). Antihypertensive effect is one of the most important
314 properties attributed to bioactive peptides, which is frequently mediated by
315 inhibition of ACE. In the last years, the study of bioactive peptides is increasing
316 due to the great variety of foods in which they are found and their use in the
317 revaluation of by-products (Margalef et al., 2017; Mora et al., 2014). In a previous
318 study, our group demonstrated that enzymatic protein hydrolysis of WL was a
319 useful methodology to maximize the extraction of phenolic compounds and to
320 obtain extracts with enhanced bioactivities, including ACEi and antihypertensive
321 properties. Moreover, since an increase in the amino acid content was also
322 observed after the hydrolysis of WL, the potential involvement of bioactive
323 peptides in BP-lowering effect of the hydrolysate was not ruled out (López-
324 Fernández-Sobrino, Margalef, et al., 2021). Therefore, in the present study, we
325 investigated the presence of antihypertensive peptides in the WL hydrolysate.

326 The biological activity of the peptides present in different protein hydrolysates is
327 related to their composition, their amino acid sizes and sequence and their
328 configuration (Möller et al., 2008). In order to the identify potential bioactive
329 peptides, WL hydrolysate was firstly ultrafiltered to obtain the smaller fraction of
330 3 kDa since peptides with the highest ACEi activity have been usually reported
331 to be short in length (between 2-11 residues) (Margalef et al., 2017). In fact,
332 fraction < 3kDa is usually used to identify ACEi peptides in protein hydrolysates

333 (Bravo et al., 2019; Quirós et al., 2007). This fraction was further fractioned using
334 RP-HPLC to separate the peptides according to their sizes and hydrophilicities.
335 Subsequently, ACEi properties of the fractions were determined to select those
336 most active for peptides identification by mass spectrometry. Six amino acid
337 sequences were identified (FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA,
338 PAGELHP, LDSPSEGRAPG and LDSPSEGRAPGAD), which were released
339 during WL protein hydrolysis since no peptides were detected in non-hydrolyzed
340 WL. The identified peptides showed a wide range of ACEi activities from 2.4 and
341 95.4 % at the same concentration (0.83 mg/mL). It is known that ACEi activity is
342 dependent on the amount and type of amino acid composition of peptides. Short
343 amino acid sequences between 2-12 residues are usually related with the best
344 ACEi activities (Daskaya-Dikmen et al., 2017). However, amino acid composition
345 seems to be more important than peptide length for ACE inhibition, in di and
346 tripeptides overall. In this regard, the amino acid sequence, concretely the three
347 last amino acid positions in the C-terminal extreme, plays an important role in
348 ACE competitive inhibition (Murray & FitzGerald, 2007). Peptides containing Pro
349 and hydrophobic amino acids (Tyr, Trp or Phe) in that extreme have been shown
350 to exert the highest ACEi activity. In fact, the presence of Pro at the last and
351 second last positions is very common in ACE inhibitory peptides. Moreover, Gly,
352 Val, Leu and Ile at the first position of N-terminal extreme or the presence of
353 positively charge amino acids (Arg or Lys) in the N-terminal amino acid sequence
354 have been linked with high ACE inhibitions (Aluko, 2015; Asoodeh et al., 2016).
355 In our study, peptides P3, P4 and P5 (TVTNPARIA, PAGELHP and
356 LDSPSEGRAPG) showed the greatest ACEi activities. The bioactivity of these
357 peptides could be due to the fact that they are small peptides, between 7-11

358 amino acids and they contained Pro and other hydrophobic amino acids at the C-
359 terminal position and/or, in the case of P5 Leu at the N-terminal position for. To
360 the best of our knowledge, none of the six identified peptides have been
361 previously identified to have any bioactivity (search carried out in the BIOPEP
362 database as of March 2021,
363 http://www.uwm.edu.pl/biochemia/index.php/pl/biopep). In addition, this is the
364 first time that ACEi peptides are identified in WL. Alcaide-Hidalgo et al. reported
365 that ACEi activity showed by red wines aged with lees could partially be mediated
366 by peptides released during the wine ageing. However, peptides were not
367 characterized (Alcaide-Hidalgo et al., 2008). Moreover, the results found in our
368 study are relevant since 3 peptides with a great potential to inhibit ACE were
369 identified. IC_{50} values showed by peptides P3, P4 and P5 were between 0.5 and
370 18 μM. These values were similar to those presented by other peptides,
371 recognized as good ACE inhibitors, such as the peptide RDGGYCC founded in
372 virgin olive oil with 0.84 μM (Alcaide-Hidalgo et al., 2020) and the peptides IPP,
373 VPP and LHLPLP with 5, 9 and 5.5 μM, respectively obtained from fermented
374 milk (Iwai et al., 2008; Quirós et al., 2007) or much higher than the peptide
375 AVFQHNCQE from a chicken foot hydrolysate (44.8 μM) (Bravo et al., 2019). All
376 these reported peptides showed antihypertensive effects in SHR after their acute
377 administration. Thus, P3, P4 and P5 could be considered good ACE inhibitors
378 and potentially could exert antihypertensive effects.

379 However, since *in vitro* ACE inhibition and antihypertensive properties are not
380 necessarily directly related (Margalef et al., 2017), it was important to evaluate
381 the *in vivo* efficacy of these peptides. In this regard, when the peptides are orally
382 consumed they can be hydrolyzed during the GI digestion due to the action of the

383 enzymes pepsin, trypsin, α-chymotrypsin, elastases, and carboxypeptidase A
384 and B or other intracellular peptidases. Therefore, peptide bioactivity could get
385 lost or also be increased depending on the bioactivity showed by the generated
386 peptide-derived fragments. In addition, the length of the peptides is very important
387 to be absorbed for intestinal cells. Small peptides (< 6 amino acids) can pass
388 through enterocytes, decreasing their absorption efficiency as the peptides are
389 longer. Furthermore, their charge and hydrophobicity play an important role in
390 their absorption by enterocytes (Fan et al., 2019). Considering all these facts, it
391 is the great relevance to evaluate the bioactivity of these peptides *in vivo*.

392 All six identified peptides were synthetized and their antihypertensive effects
393 were evaluated in SHR at an acute and oral dose of 10 mg/kg bw. All peptides
394 showed an antihypertensive effect in both SBP and DBP, except for P4
395 (PAGELHP) that did not show this bioactivity. Peptides P2, P3 and P5 produced
396 a reduction in SBP between -16 or -19 mmHg with the maximum BP-reduction at
397 6 h post-administration. P1 and P6 showed the highest effect, with a SBP
398 reduction to -27.6 and -24.3 mmHg, respectively and with similar results in DBP
399 after 6 h post-administration. Similar BP- lowering effects have been found in
400 purified peptides obtain from dietary food (Alcaide-Hidalgo et al., 2020; Bravo et
401 al., 2019; Shobako et al., 2018). Thus, AVFQHNCQE peptide administration to
402 SHR at the same concentration used in this study (10 mg/kg bw) showed a
403 maximum BP reduction at 6 h post-administration by -25.07 ± 4.21 mmHg and
404 −17.65 ± 3.24 mmHg in SBP and DBP, respectively, similar to the peptides
405 obtained from WL hydrolysate (Bravo et al., 2019). It is well known that a
406 reduction by 10 mmHg and 5 mmHg in SBP and DBP respectively, leads to
407 healthy effects including the reduction of cardiovascular diseases by 25% and

408 stroke by 36 % (Law et al., 2009). Thus, these results show the potential of the
409 peptides P1, P2, P3, P5 and P6 to manage hypertension. It should be noted that
410 the peptide P4, which showed the higher ACEi activity, did not have
411 antihypertensive effect. This fact was also observed for other peptides from a
412 chicken foot hydrolysate with high ACEi activities such as VGKPGARAPMY,
413 LSGPVKF and AVKILP, that did not showed antihypertensive effects *in vivo*
414 (Bravo et al., 2019). In addition, the peptide P6, with the lowest ACEi activity,
415 showed the best antihypertensive effect. This could be indicative that other
416 peptides could be generated during GI digestion.

417 Digestion *in silico* studies have shown to be a good tool to predict the possible
418 protein hydrolysis produced by GI enzymes (Mas-Capdevila et al., 2020). All the
419 peptides identified in this study were susceptible to GI hydrolysis by trypsin,
420 chymotrypsin or pepsin, generating other amino acid sequences which could be
421 responsible of the bioactivity of these peptides identified in hydrolyzed WL. This
422 fact could explain for example why P6 did not have a good ACEi activity but
423 exhibited antihypertensive effect. Moreover, it was observed that the peptide
424 LDSPSEGRAPGAD produced a higher decrease in BP compared to the peptide
425 LDSPSEGRAPG, which have a similar sequence except for missing two amino
426 acids at its C-terminal (AD). Digestion of the largest peptide
427 (LDSPSEGRAPGAD) did not produce the smallest peptide but similar peptide-
428 derived fragments were generated after *in silico* digestion in both. Therefore, the
429 greatest antihypertensive effect is probably due to the terminal amino acids AD
430 in the peptide LDSPSEGRAPGAD. However, the *in silico* study is a predictive
431 model that does not take into account the tertiary structure of proteins; therefore,
432 some of the predicted amino acid sequences may not be generated by an *in vivo*

433 GI digestion. The sequences obtained after *in silico* digestion were also analysed
434 using the BIOPEP database to consult if these amino acid sequences have been
435 already reported as ACE inhibitors and/or antihypertensive sequences. None of
436 the peptides or their predicted GI-derived amino acid sequences were found in
437 this database having those activities except for the dipeptides IA and HP. These
438 two peptides had been reported as ACE inhibitors. Therefore, further studies are
439 required to demonstrate which amino acid sequences could be responsible of the
440 antihypertensive activity of the peptides identified in this WL hydrolysate.

441 **5. Conclusions**

442 The hydrolysis of WL under specific conditions produced the release of peptides
443 with antihypertensive effect. BP-lowering effects of the novel peptides
444 FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, LDSPSEGRAPG,
445 LDSPSEGRAPGAD were shown in SHR. For the first time, it was demonstrated
446 that WL can be source of ACE inhibitory and antihypertensive peptides. These
447 results confirm the potential of these compounds to be used as functional
448 ingredient or nutraceuticals to treat HTN.

449 **6. Patents**

450 Patent application “Wine lees, derivatives thereof and their uses”: application
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452 **Author Contributions:** Conceptualization, B.M. and F.I.B.; Formal analysis, R.L-
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454 M.M.; Investigation, R.L-F-S., A.M-C, J.M.A-H.; Methodology, R.L-F-S. A.M-C,
455 J.M.A-H., F.I.B; Supervision, B.M., F.I.B. and J.M.A-H.; Writing—Original Draft,

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475 **Conflicts of Interest:**

476 The authors declare no conflict of interest.

477

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630 **TABLES**

631 **Table 1.** Amino acid sequences identified by reverse phase liquid
632 chromatography, percentage of purity and angiotensin-converting enzyme
633 inhibitory (ACEi) activity of the peptides identified in the wine lees hydrolysate.
634 Significant differences ($p < 0.05$) between peptides in the ACEi activity are
635 represented by different letters. *p* value was estimated by one-way ANOVA and
636 Tukey test was used as post hoc.

	Sequence[1]	Theoretical M.W.	MH+ (Da)	m/z (Da)	Charge	Purity grade (%)	ACEi activity	
							%[2]	IC_{50} (µM)[3]
P1	FKTTDQQTRTTVA	1496.65	1496.77	748.89	2	97.8	27.0 ± 5.6[a]	N.D
P2	NPKLVTIV	883.11	883.56	413.75	2	99.2	48.5 ± 9.1[b]	N.D
P3	TVTNPARIA	942.09	942.53	471.77	2	96.4	76.4 ± 9.2[c]	14.5
P4	PAGELHP	719.80	720.37	360.68	2	99.4	72.3 ± 11.5[c,d]	0.6 ± 0.2
P5	LDSPSEGRAPG	1085.15	1085.52	543.26	2	96.9	95.4 ± 2.2[c,e]	16.4 ± 1.0
P6	LDSPSEGRAPGAD	1271.32	1271.59	636.29	2	98.5	2.4 ± 0.9[f]	N.D

637 [1] Amino acids are designated using their one letter codes.
638 [2] ACEi activity determined at 0.83 mg/mL.
639 [3] Peptide concentration needed to inhibit 50 % of the original ACE activity N.D= no determined
640

641 **Table 2.** *In silico* simulated digestion of the peptides P1-P6 identified in the wine

642 lees hydrolysate.

Peptide	Original Sequence	Enzyme	Digestion Stage	Final Sequence	ACE inhibitor
P1	FKTTDQQTRTTVA	Trypsin	Duodenal	TTDQQTRTTVA	
	FKTTDQQTRTTVA	Trypsin	Duodenal	FK	FK-
	TTDQQTRTTVA	Trypsin	Duodenal	TTDQQTR	
	TTDQQTRTTVA	Trypsin	Duodenal	TTVA	
P2	NPKLVTIV	Pepsin	Gastric	NPKL	-KL
	NPKLVTIV	Pepsin	Gastric	VTIV	
	NPKLVTIV	Chymotrypsin	Duodenal	NPKL	-KL
	NPKLVTIV	Chymotrypsin	Duodenal	VTIV	
	NPKLVTIV	Trypsin	Duodenal	NPK	
	NPKLVTIV	Trypsin	Duodenal	LVTIV	
	NPKL	Trypsin	Duodenal	NPK	
	LVTIV	Chymotrypsin	Duodenal	VTIV	
P3	TVTNPARIA	Trypsin	Duodenal	TVTNPAR	-AR
	TVTNPARIA	Trypsin	Duodenal	IA	IA
P4	PAGELHP	Pepsin	Gastric	PAGE	-GE
	PAGELHP	Pepsin	Gastric	LHP	LHP-, -HP
	PAGELHP	Chymotrypsin	Duodenal	PAGEL	
	PAGELHP	Chymotrypsin	Duodenal	HP	HP
	LHP	Chymotrypsin	Duodenal	HP	HP
P5	LDSPSEGRAPG	Pepsin	Gastric	DSPSEGRAPG	
	LDSPSEGRAPG	Chymotrypsin	Duodenal	DSPSEGRAPG	
	LDSPSEGRAPG	Trypsin	Duodenal	LDSPSEGR	-GR
	LDSPSEGRAPG	Trypsin	Duodenal	APG	APG-, -APG
	DSPSEGRAPG	Trypsin	Duodenal	DSPSEGR	-GR
	DSPSEGRAPG	Trypsin	Duodenal	APG	APG-, -APG
	LDSPSEGR	Chymotrypsin	Duodenal	DSPSEGR	-GR
P6	LDSPSEGRAPGAD	Pepsin	Gastric	DSPSEGRAPGAD	
	LDSPSEGRAPGAD	Chymotrypsin	Duodenal	DSPSEGRAPGAD	
	LDSPSEGRAPGAD	Trypsin	Duodenal	LDSPSEGR	-GR

LDSPSEGRAPGAD	Trypsin	Duodenal	APGAD	APG-
DSPSEGRAPGAD	Trypsin	Duodenal	DSPSEGR	-GR
DSPSEGRAPGAD	Trypsin	Duodenal	APGAD	APG-
LDSPSEGR	Chymotrypsin	Duodenal	DSPSEGR	-GR

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FIGURES

Figure 1. A) Fractionation by RP-HPLC at a semipreparative scale for the <3 kDa fraction obtained from the wine lees hydrolysate. The collected fractions were termed with F. followed by a number (F.1–F.9). Angiotensin-converting enzyme inhibitory (ACEi) activity expressed as B) percentage of inhibition and C) IC_{50} (μgmL^{-1}) of the collected fractions from semipreparative RP-HPLC system. Data are expressed as the mean ± SD (n=3). Protein contents of fractions were estimated using the bicinchoninic acid assay.

Figure 2. Changes in systolic blood pressure (SBP) in spontaneously hypertensive rats after administration of water, Captopril (50 mg/kg bw) or synthetic peptides (10 mg/kg bw): FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, PAGELHP, LDSPSEGRAPG, LDSPSEGRAPGAD. Data are expressed as the mean ± SEM ($n \geq 6$). Different letters (a, b or c) represent significant differences ($p < 0.05$). p was estimated by two-way ANOVA.

Figure 3. Changes in diastolic blood pressure (DBP) in spontaneously hypertensive rats after administration of water, Captopril (50 mg/kg bw) or synthetic peptides (10 mg/kg bw): FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, PAGELHP, LDSPSEGRAPG, LDSPSEGRAPGAD. Data are expressed as the mean ± SEM ($n \geq 6$). Different letters (a, b or c) represent significant differences ($p < 0.05$). p was estimated by two-way ANOVA.